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The Influence of Lutein on Swine Embryo Development

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Abstract. As defence against oxidative stress, living systems employ antioxidants that they either produce or take up from the environment. Among these lutein has been shown to be a powerful antioxidant that also functions as a signal molecule. The goal of this study was to establish the influence of several lutein concentrations on swine embryo development in vitro, in order to improve culture media. Pig oocytes were cultured for 45 hours at 37°C in 5% CO_2 atmosphere; in M199 containing several lutein (2.5, 4, 5, 8, and 10 µM) concentrations. Then, they were fertilized in TALP medium using spermatozoa capacitated by centrifugation and incubated for 16-18 hours. Afterwards, the presumed zygotes were cultured in NCSU-23 droplets supplemented with lutein (2.5, 4, 5, 8, and 10 µM) in the same conditions. Their development was assessed at 48 and 120 hours. The number of embryos that had developed to the 2 cells, 4-8 cells and morula stages was compared to the control, and the differences analyzed using the analysis of variance and interpreted using the LSD and Duncan tests. Lutein (2.5 µM) supplementation has a beneficial effect on embryo development to the morula stage. This is apparent at 48 hours as well as 120 hours.

Keywords: lutein, concentration, antioxidant, pig, embryo

INTRODUCTION

Although, the in vitro production of swine embryos has been greatly improved by the development of NCSU-23, NCSU-37 and their subsequent modifications it is still not as efficient as its in vivo counterpart. Very high levels of lipids have been reported in the oocytes of the domestic pig -161 µg (McEvoy *et al.,* 2000). Triacylglycerol was the major lipid component followed by cholesterol and phosphatidylcholine. Analysis of fatty acids esterified to the individual phospholipids and neutral lipids has shown that there are high levels of palmitic acid (16:0) and the monounsaturated fatty acid oleic acid (18:1). Triacylglycerol, free fatty acids and most of the phospholipids, particularly phosphatidylethanolamine, are considerably enriched in n-6 polyunsaturated fatty acids, specifically linoleic (18:2), arachidonic (20:4) and adrenic (22:4) acids (Homa *et al.*, 1986).

The majority of these lipids are transferred into the embryo where they serve as energy sources and/or are utilized for the biosynthesis of membrane components. The total volume of the lipid droplets remains constant from the zygote to the morula stage and then decreases in the blastocyst and late blastocyst stages (Romek *et al.,* 2010). Also, during development the amount of unsaturated lipids, free fatty acids, and phospholipids decreases (Romek *et al.,* 2009).

In the cell, free radicals function as signal molecules by activating transcription factors and enzymatic reactions (Droge, 2002). In addition, they play a significant role in steroidogenesis, follicle formation and progesterone release by the corpus luteum and together

with antioxidant enzymes bring about follicle wall rupture, ovulation and luteal regression (Agarwal *et al.*, 2006). They are also involved in regulating embryo development and implantation (Guerin *et al.*, 2001).

Although cells have various antioxidant systems that should scavenge endogenous free radicals, their endogenous overproduction and the exogenous sources lead to an imbalance in redox metabolism and therefore to oxidative stress. It causes chain reactions that result in mitochondrial depolarization, cytochrome c release, lipid peroxidation, transcription factor activation and DNA damage leading to apoptotic and non-apoptotic cell death.

Protection of the fatty acid and lipid components of oocytes and embryos that render them susceptible to free radical or other oxidative injury may prevent the damage currently associated with in vitro culture.

According to Halliwell and Gutteridge (2007) antioxidants are "any substance that delays, prevents or removes oxidative damage to a target". The definition applies to enzymes as well as non enzymatic antioxidants. To defend themselves, living systems employ antioxidants that they either produce or take up from the environment.

Lutein, part of the xanthophylls class of carotenoids is one of the most potent lipid soluble antioxidant. It can be found in the retina and macula lutea (Stahl and Sies, 2005) and is readily incorporated in membrane bilayers of mitochondria and microsomes (Chew and Park, 2004). Retinoids, which are carotenoid metabolites have been shown to increase the number and the improve quality of swine embryos (Brines, 2008). Both types of substances are antioxidants and function as signal molecules that modulate cholesterol metabolism and cell adhesion.

The objective of this research was to establish whether supplementation with lutein could improve viability and in vitro development of porcine embryos.

MATERIALS AND METHODS

Collection, maturation, fertilization and embryo culture media. The oocyte collection medium was M 199 supplemented with L-glutamine (3.4 g/l), $NaHCO_3$ (2.2 g/l), Hepes (25 mM), penicillin (100 µg/ml) and streptomycin (100 IU/ml) and with the pH adjusted to 7.

For oocyte maturation M 199 was supplemented with L-glutamine (3.4 g/l), Chorulon (10 IU/ml), Folligon (10 IU/ml), foetal calf serum 10%, penicillin (100 µg/ml) and streptomycin (100 IU/ml). Lutein dissolved in foetal bovine serum was added to the maturation medium in order to arrive at concentrations of 2.5, 4, 5, 8, and 10 µM carotenoid and 10% foetal bovine serum.

The fertilization medium was TALP, which is based on Tyrode's saline solution supplemented with bovine serum albumin (0.004 g/l), glucose (1.00 g/l), sodium lactate (10 µl/ml), sodium pyruvate (0.288 µg/ml) and antibiotics (100 µg/ml penicillin and 100 IU/ml streptomycin). The embryo culture medium was NCSU-23 to which sodium lactate (4.5 mM), sodium pyruvate (0.33 mM), β-mercaptoethanol (25 µM), cysteine (0.1 mg/ml), bovine serum albumin (4 mg/ml) and antibiotics (75 µg/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin) were added. Lutein dissolved in foetal bovine serum was added to the maturation medium in order to arrive at concentrations of 2.5, 4, 5, 8, and 10 µM carotenoid and 10% foetal bovine serum.

Oocyte collection and maturation. Porcine ovaries were collected from pre-pubertal gilts and transported to the laboratory in a thermal container containing sterile saline solution (NaCl 0.9%) at 37°C supplemented with penicillin (100 µg/ml) and streptomycin (100 IU/ml). The contents of follicles of 2–6 mm in diameter on the ovarian surface were aspired

with a 10 ml syringe equipped with an 21-gauge needle and collected a Petri dishes containing harvest medium. Oocytes with a uniform ooplasm and compact cumulus cell mass were washed 2 times with harvest medium and then placed in 30 µl droplets of maturation medium containing the various lutein concentrations. All the droplets were covered in paraffin oil and incubated for 45 hours at 37°C in an atmosphere with 5% CO_2.

In vitro fertilization and embryo culture. Spermatozoa were capacitated in Tyrode by centrifugation at 800 g for 10 minutes, 3 times and a haemocytometer was employed to establish concentration. After maturation oocytes were transferred into TALP and mechanically denuded using a micropipette. Afterwards they were placed in 30 µl droplets and the sperm cells were added. The concentration for fertilization was $1x10^6$ spermatozoa/ml and it was computed according to the following formula: volume (µl) = $30x1x10^6$ /the concentration of capacitated spermatozoa. After the sperm cells were added the droplets were covered with paraffin oil to prevent media evaporation and incubated at 38°C and 5 % CO_2 in air. After 16-18 hours the spermatozoa were removed mechanically using a micropipette and the presumed zygotes were cultured in NCSU-23 droplets supplemented with lutein (2.5, 4, 5, 8, and 10 µM) and covered with mineral oil.

Embryo development was assessed at 48 and 120 hours after fertilization. The number of embryos that had developed to the 2 cells, 4-8 cells and morula stages was counted and compared to the control, and the differences analyzed using the analysis of variance and interpreted using the LSD and Duncan tests. For all comparisons, the values were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

The goal of this study was to establish the influence of several lutein concentrations (2.5, 4, 5, 8, and 10 µM) on swine embryo development. Embryos were assessed at 48 and 120 hours. These two moments were chosen because of the slower development rate of in vitro produced swine embryos. They also helped us understand the influence of lutein on embryo development dynamics.

In lutein supplemented NCSU-23, embryos developed at a rate of 50.60%. At 48 hours the percentage of embryos in the two cell stage was higher than the control for all lutein concentrations. Differences were statistically significant ($p<0.05$) for two out of the five concentrations, namely 4 µM and 8 µM.

The percentages of embryos that had reached the 4-8 cell stage were below the value of the control (56.48%) for all employed concentrations and the differences were significant in two instances (Tab. 1).

More embryos developed to the morula stage in NCSU-23 supplemented with 2.5 µM, 8 µM or 10 µM lutein than 4 µM or 5 µM lutein. Thisfact is strengthened by the fact that differences between the control and the first concentration were significant ($p<0.05$). Thus, it becomes apparent that the addition of 2.5 µM lutein has a beneficial influence on swine embryo development to the morula stage.

Percentages for the total number of embryos were lower than the control without being significant.

Tab. 1

Percentages of embryos at different cleavage stages, at 48 hours after fertilization

Treatment	Number of oocytes	Cultured embryos (%)	Percentages of embryos at each development stage		
			2 cells	4-8 cells	Morula
Control	167	58.22	25.83	56.48	17.69
Lut 2.5	131	46.71	36.20	33.51 (°)	30.29 (**)
Lut 4	161	44.85	45.63 (*)	45.54	9.16
Lut 5	194	48.26	41.13	45.97	12.90
Lut 8	168	55.46	53.85 (**)	24.15 (°°)	22.01
Lut 10	175	52.93	36.56	45.01	18.43

*significant and positive ($p<0.05$); **distinctly significant and positive ($p<0.01$);
°significant and negative ($p<0.05$); °°distinctly significant and negative ($p<0.01$)

At 120 hours significantly more embryos were in the 2 cell stage when cultured in 4 µM lutein (Tab. 2). The differences are less significant then they were at 48 hour analysis. This indicates that embryos continued their development to superior stages.

Tab. 2

Percentages of embryos at different cleavage stages, at 120 hours after fertilization

Treatment	Number of oocytes	Percentages of embryos at each development stage		
		2 cells	4-8 cells	Morula
Control	167	29.65	37.55	25.73
Lut 2.5	131	34.51	35.05	30.44
Lut 4	161	48.34 (*)	32.74	18.93
Lut 5	197	36.91	42.37	20.72
Lut 8	168	38.20	43.58	18.22
Lut 10	175	28.67	50.35	20.98

*significant and positive ($p<0.05$)

Percentages indicate that the majority of the embryos were at the 4-8 cell stage, the best concentration being 80 µM.

Out of the five concentrations 4 µM, 5 µM, 8 µM and10 µM did not bring about an increase in the percentages of embryos that developed to the morula stage. However, 2.5 µM did prove beneficial to embryo development. This suggests that there are optimal concentrations at which such substances are beneficial and that these are not the highest.

The effect can be ascribed to lutein antioxidant activity that can be observed in the presence of light induced free radicals (Stahl and Sies, 2005). It is also worth noting that lutein has been known to modulate the expression of genes encoding connexins, which are the most important proteins in gap junction formation (Bertram, 2004). These function as the foremost means of substance transfer between the cells that make up the embryo. Therefore, lutein enhances intercellular communication.

CONCLUSIONS

Lutein supplementation has a beneficial effect on embryo development to the morula stage, the optimal concentration being 2.5 µM. This is apparent at 48 hours as well as 120 hours. Other concentrations such as 4 µM and 5 µM increased the number of embryos in the 2 cell stage.

The majority of embryos cultured in lutein supplemented medium developed to the morula stage, while in its absence embryos remained in the 2 cell and 4-8 cell stage.

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